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Cometabolic enzymatic transformation of organic micropollutants under methanogenic conditions

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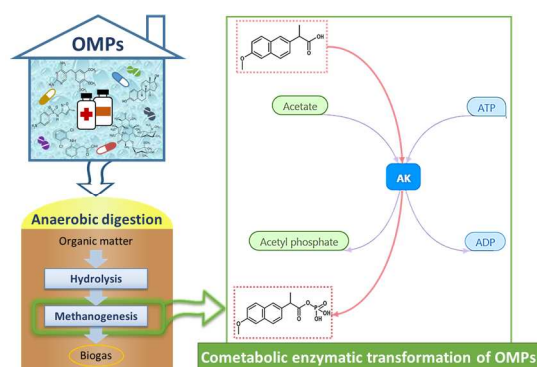
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Keywords: acetate kinase, anaerobic digestion, biotransformation, cometabolism, organic micropollutants, sewage sludge.

ABSTRACT

Anaerobic digestion (AD) has been shown to have the biological potential to decrease the concentrations of several organic micropollutants (OMPs) from sewage sludge. However, the mechanisms and factors behind these biotransformations, which are essential for elucidating the possible transformation products and to foster the complete removal of OMPs via operational strategies, remain unclear. Therefore, this study investigated the transformation mechanisms of 20 OMPs during the methanogenic step of AD with a focus on the role of acetate kinase (AK), which is a key enzyme in methane production. The results from lab-scale methanogenic reactors showed that this step accounts for much of the reported OMPs biotransformation in AD. Furthermore, enzymatic assays confirmed that AK transforms galaxolide, naproxen, nonylphenol, octylphenol, ibuprofen, diclofenac, bisphenol A and triclosan. Except for galaxolide, for which further studies are required to refine conclusions, the OMP's chemical structure was a determinant for the AK action because only compounds that contain a carboxyl or a hydroxyl group and that have a moderate steric hindrance were enzymatically transformed, likely by phosphorylation. For these 7 compounds, this enzymatic mechanism accounts for 10-90% of the measured methanogenic biotransformation, suggesting that other active enzymes of the AD process are also involved in OMPs biotransformation.

Abstract Art



INTRODUCTION

The increasing use of pharmaceuticals, personal care products, hormones and many other organic compounds in our daily life has resulted in the release of these organic micropollutants (OMPs) into the environment via diverse pathways.¹ There is evidence of negative effects of OMPs on human and ecosystem health,² which suggests that reduction measures should be applied, especially for one of the main sources of OMP discharge: effluents of sewage treatment plants (STPs).

Anaerobic digestion (AD) is widely used in STPs for sludge stabilization prior to its application in agricultural soils as a biosolid. Significant quantities of OMPs arrive at the sludge treatment line sorbed onto solids³ but also solubilized in the water phase of the sludge, achieving concentrations up to 90-140 $\mu\text{g L}^{-1}$ for musk fragrances (galaxolide and tonalide), 40 $\mu\text{g L}^{-1}$ for triclosan, 25 $\mu\text{g L}^{-1}$ for ibuprofen, and 1-10 $\mu\text{g L}^{-1}$ for hormones (estrone and 17 β -estradiol).⁴ Most of these OMPs remain in the digested sludge,⁴ thus its use as a fertilizer might transfer OMPs to the soil.⁵

Despite the environmental risks associated with biosolid-amended soils,⁵ few studies have investigated the fate of OMPs during AD.^{4,6-12} They conclude that AD is able to biologically transform OMPs, but the degree of removal of some compounds is still controversial, and few transformation products (TPs) have been identified, which indicates the poor understanding of the microbial mechanisms and factors behind these biotransformations.³ Therefore, to develop strategies that promote the complete elimination of OMPs and to predict the environmental risks of the TPs that are generated, it is essential to understand the biotransformation pathways involved in AD.

To date, only rare and inconclusive information is available about the relationship between anaerobic populations and the removal of OMPs.¹³ This lack of knowledge is justified by the complexity of the biological and chemical processes involved in the four steps of AD (Figure 1): hydrolysis, acidogenesis, acetogenesis and methanogenesis.¹⁴ According to the taxonomic analysis of Guo et al.,¹⁵ *Bacteria* (~93%) was more abundant than *Archaea* (methanogens) (~6%) on sewage sludge AD. Among bacteria, *Proteobacteria* (41%), *Firmicutes* (13%) and *Bacteroidetes* (10%) are the most abundant populations, while *Methanosaeta* (26%), *Methanospirillum* (13%), *Methanosarcina* (13%), *Methanoculleus* (11%) and *Methanoregula* (8%) are the dominant methanogenic genera. During the four AD steps, these microorganisms produce a broad diversity of enzymes to degrade the organic substrates. Depending on their specificity, these enzymes can also modify the structures of OMPs despite being non-growth substrates. This biochemical process is known as cometabolism, and it is considered by many authors to be a major removal mechanism of OMPs during the biological treatment of wastewater.^{16–21} The action of enzymes on OMPs is poorly investigated, and most studies have focused on oxygenases.^{21–23} A recent study by Krah et al.²⁴ tested the activity of a cocktail of extracted enzymes from activated sludge towards OMPs. By measuring several TPs and using several enzymatic inhibitors, they concluded that amide hydrolases could be involved in the biotransformation of acetaminophen, acetyl sulfamethoxazole, atenolol and bezafibrate, that oxidoreductases transform 10-OH-carbamazepine and that erythromycin is affected by hydrolases acting on ester bonds. However, the suspected participation of particular enzymes in the cometabolic biotransformation of OMPs was not definitively confirmed because the identification of enzymes by indirect measurements is unreliable, and the isolation of target enzymes has not yet been reported.²¹ Although no studies about the enzymatic transformation routes of OMPs during AD have been found, it could be hypothesized that hydrolases from the

two first AD steps, which perform relatively simple reactions, are involved in the biotransformation of some OMPs, as was demonstrated for activated sludge systems.²⁴ For the more specific acetogenic and methanogenic steps, the removal of polycyclic aromatic hydrocarbons (PAH) has been linked to methanogenesis,²⁵ but the type of enzymatic biotransformation that could occur is not clear.

The acetoclastic methanogenesis (Figure 1) is performed by the archaea *Methanosaeta* and *Methanosarcina* and it is considered the main pathway in the synthesis of methane in anaerobic digesters.^{15,26} The first step in the methanization of acetate by *Methanosarcina* species is the phosphorylation of acetate to acetyl phosphate (Figure 1 and Figure S1), which is performed by the key intracellular enzyme acetate kinase (AK). Then, the acetyl phosphate is further converted to acetyl-CoA by phosphotransacetylase (PTA)²⁷ and the acetoclastic methanization continues with the action of other enzymes (Figure S1). In addition, AK associated with PTA catalyzes the reverse reaction (conversion of acetyl-CoA to acetate; Figure 1) in fermentative prokaryote bacteria during acetogenesis.²⁸ AK is a relatively specific enzyme but, in addition to acetate, it is able to phosphorylate other substrates to a lower extent;^{29,30} thus, AK might also have effects on some OMPs.

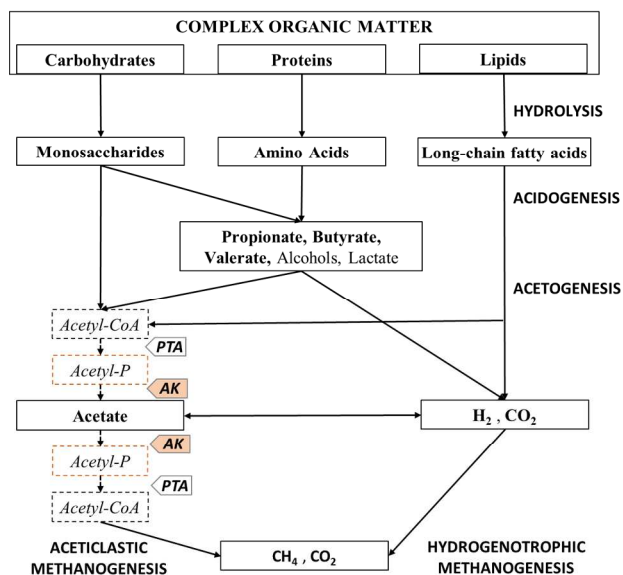


Figure 1. Schematic representation of the steps of anaerobic digestion, including the roles of the enzymes acetate kinase (AK) and phosphotransacetylase (PTA).

The major aim of this work was to gain insight into the biotransformation pathways involved in the removal of OMPs during methanogenesis. In particular, the enzymatic transformation of OMPs was assessed via in vitro assays with AK. Furthermore, the cometabolic impact of AK on the biotransformation of OMPs in methanogenic reactors (MRs) was quantified. To the best of our knowledge, this is the first attempt to clarify the role of AD enzymes on the biotransformation of OMPs.

MATERIALS AND METHODS

Organic micropollutants. This study focuses on 20 compounds that are commonly detected in sewage sludge^{3,4,8,9,31–33} and whose chemical structures (Table S1), applications and physicochemical properties (Table S2) are representative of a huge range of OMPs. The selected pollutants are: the musk fragrances galaxolide (HHCB), tonalide (AHTN) and celestolide (ADBI); the anti-inflammatories ibuprofen (IBP), naproxen (NPX) and diclofenac (DCF); the

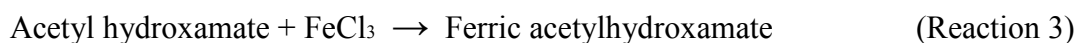
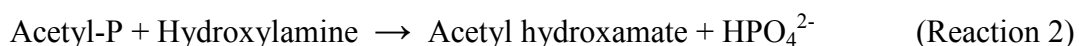
antibiotics sulfamethoxazole (SMX), trimethoprim (TMP), erythromycin (ERY) and roxithromycin (ROX); the neurodrugs fluoxetine (FLX), carbamazepine (CBZ) and diazepam (DZP); and the endocrine-disrupting compounds triclosan (TCS), bisphenol A (BPA), 4-octylphenol (OP), 4-nonylphenol (NP), estrone (E1), 17 β -estradiol (E2) and 17 α -ethinylestradiol (EE2). These substances were purchased from Sigma-Aldrich (Steinheim, Germany) except for the fragrances, which were provided by Ventos (Spain). Stock solutions were prepared in HPLC grade methanol or acetone, depending on the compound, and stored at -18 °C.

Methanogenic reactor. Two lab-scale (14 L) continuously stirred (IKA RW20, 150 rpm) MRs were operated under mesophilic (37 °C) conditions. Both reactors were inoculated with sludge from a mesophilic STP anaerobic digester. The feeding consisted of a synthetic mixture of volatile fatty acids (VFA) (acetic:butyric:propionic 50:25:25, %COD) and other trace nutrients (section S4), in order to promote the growth of acetogenic bacteria and aceticlastic methanogenic archaea (i.e. *Methanosaeta* and *Methanosarcina*).^{15,34,35} After a start-up period, both MRs reached steady-state operation at a hydraulic retention time (HRT) of 10 d, an organic loading rate (OLR) of 1 g COD L⁻¹ d⁻¹ and a methanization efficiency above 70%. After 1-2 months under these conditions (Table S4), a pulse of the selected OMPs (100 μ g L⁻¹ except for the hormones, which were 10 μ g L⁻¹, section S4) was added to each MRs, and their concentrations were followed in the liquid (17 samples) and solid (10 samples) phases for 10 d.

Acetate kinase activity. Acetate kinase (AK, EC 2.7.2.1) is a homodimer with two active sites that catalyze the reversible Mg-dependent transfer of the γ -phosphoryl group from adenosine triphosphate (ATP) to acetate.²⁷ AK from *Methanosarcina thermophila* was chosen for this study because it is a well characterized and investigated enzyme²⁹ and because *Methanosarcina* species appear to be key organisms in AD.³⁶ AK from *M. thermophila*

recombinant, expressed in *E. coli*, was purchased from Sigma-Aldrich (USA) as lyophilized powder (885 U mg⁻¹ protein (Bradford) and 6.94 U mg⁻¹ solid). One unit (U) phosphorylates 1.0 μmol of acetate to acetyl phosphate per min at pH 7.6 and 25 °C. The AK powder was stored at -20 °C. Immediately before use, a solution of AK (288 μg mL⁻¹, 2.0 U mL⁻¹) in a potassium phosphate buffer (0.1 M, pH 7.4) was prepared.

Hydroxamate assay. The hydroxamate assay^{37,38} is the simplest and most convenient method to determine the AK activity in the direction of acetyl phosphate synthesis.²⁹ This standard assay measures the rate of the forward Reaction 1 in the presence of hydroxylamine, which reacts with acetyl phosphate (Reaction 2) to form a colored complex in the presence of trivalent iron (Reaction 3). Under standard conditions, dephosphorylate acetyl phosphate is thermodynamically more favorable (Reaction 1); therefore, hydroxylamine is required to shift the reaction equilibrium to the right through the removal of acetyl phosphate.³⁷



An adaptation of the hydroxamate assay³⁰ was used in this study. A stock solution was prepared with the following components: 290 mM of Tris-HCl (pH 7.4, neutralized with KOH), 400 mM of potassium acetate, 20 mM of MgCl₂·6H₂O, 20 mM of ATP and 1410 mM of hydroxylamine hydrochloride (pH 7.4, neutralized with KOH). This reaction mixture was prepared immediately before use, since the aqueous solution of ATP at room temperature and the neutralized hydroxylamine solution have a limited stability. The reaction started by adding 1.0 mL of the enzyme solution over 1.0 mL of the pre-warmed (25 °C) reaction mixture. After

incubation for 12 min at 25 °C, the reaction was stopped by the addition of 2.0 mL of 10% trichloroacetic acid. Then, the color reaction was initiated by adding 2.0 mL of FeCl₃ (2.5% in 2 N HCl). After 5-30 min of incubation to allow for the formation of the colored complex,³⁸ the absorbance was recorded spectrophotometrically (Cecil CE-7200, UK) at 540 nm.

A standard curve was prepared by adding 0-1.7 U mL⁻¹ of AK to the reaction mixture following the previously described methodology (Figure S2). Furthermore, because 1 U of enzyme dephosphorylates 1 μmol of ATP per minute, the consumption of ATP (μmol mL⁻¹) can also be correlated with the measured absorbance (Equation S1).

AK activity in the methanogenic reactor. The AK activity inside the reactor was determined without special precautions to avoid the presence of air (AK is not sensitive to oxygen)^{30,39} following a methodology adapted from Mu et al.⁴⁰ Three samples 25 mL were withdrawn at different time points after the OMPs spike (10 min, 3 d and 10 d) and then centrifuged at 3107 g for 15 min. The supernatant was discarded, and the biomass was washed and resuspended in 25 mL of 0.1 M sodium phosphate buffer (pH 7.4). This procedure was repeated three times. The last resuspended mixture was sonicated at 20 kHz and 4 °C for 10 min to break down the cell walls and release the intracellular AK. The sample was then centrifuged at 15344 g and 4 °C for 30 min to remove the waste debris. When needed, the extracts were stored at -20 °C before measuring the enzyme activity assay via the hydroxamate assay.

Experiments with commercial AK. *Preliminary assays.* The relevance of several key parameters (temperature, pH and OMPs concentration) on the AK activity was first evaluated because they could impair the possible action of AK over the OMPs. Studies with AK from *M. thermophila* have typically been performed at 37 °C,^{27,30,41,42} but the product information from SIGMA recommends a temperature of 25 °C for the enzymatic assay. Therefore, several

theoretical AK activities (0.01, 0.05, 0.2 U mL⁻¹) were evaluated via the hydroxamate assay at both temperatures. The results did not show significant differences (Table S5); thus, the lower temperature (25 °C) was chosen for the next assays to minimize possible activity losses by enzyme denaturation in long-term experiments.⁴³

A similar procedure was followed for the pH selection. According to Aceti and Ferry³⁰ and the product information from SIGMA, the maximum activity of AK appears between pH 7.0-7.6. Both extreme pHs were tested and, as expected, the effect on the AK activity (0.02 U mL⁻¹) was negligible (Table S5). To prevent the pH from decreasing below 7.0 during the OMP assays due to the consumption of acetate (weak base), the initial pH was adjusted with KOH to 7.6.

Finally, a possible inhibition of the AK activity by the OMPs was examined at 3 OMPs/AK ratios: 0.35 µg mg⁻¹ (50 µg OMPs L⁻¹ and 144 mg AK L⁻¹), 3.5 µg mg⁻¹ (100 µg OMPs L⁻¹ and 28.8 mg AK L⁻¹) and 69 µg mg⁻¹ (100 µg OMPs L⁻¹ and 1.44 mg AK L⁻¹). In all cases, a negative control without OMPs was included. The results (Table S5) indicated that AK activity was not altered by any OMPs/AK ratio tested, and thus neither by the corresponding solvents added with the OMPs pulse. The OMPs/AK ratio employed in the OMPs assays was 35 µg mg⁻¹ (based on the theoretical AK activity of a mesophilic AD operating at an OLR of 2 g COD L⁻¹ d⁻¹ with a 70% methanization efficiency).

Enzymatic transformation of OMPs. Once the operating parameters were selected (25 °C, pH 7.6, 100 µg L⁻¹ of OMPs and 2.9 mg L⁻¹ of AK), the enzymatic transformation of the OMPs was evaluated at two reaction times (1 d and 5 d) with and without the primary substrate (acetate) in duplicate in 100 mL Erlenmeyer flasks. The final reaction media contained 145 mM of Tris-HCl, 10 mM of MgCl₂·6H₂O, 705 mM of hydroxylamine hydrochloride, 200 mM of potassium acetate (in excess) and 50 mM of ATP. The acetate concentration, ATP consumption (Equation

S1), AK activity, pH and temperature were monitored (n=2) at different reaction times (5-11 time points) and the concentration of OMPs was measured in replicate at the beginning (n=4) and end (n=4) of each experiment.

Abiotic disappearance of OMPs. The disappearance of the parent micropollutants by adsorption was evaluated by comparing the concentration of OMPs ($100\text{ }\mu\text{g L}^{-1}$ in distilled water) after 1 d at $25\text{ }^{\circ}\text{C}$ in contact with AK (2.9 mg L^{-1}) and without the enzyme. No representative differences were found (data not shown), so the adsorption of OMPs during the enzymatic assays with commercial AK was dismissed. Furthermore, to discard possible losses of the parent compound by evaporation, analytical difficulties or chemical reaction with the media (145 mM of Tris-HCl, 10 mM of MgCl_2 , 705 mM of hydroxylamine hydrochloride, 50 mM of ATP, 200 mM of potassium acetate), duplicated negative controls (without AK) were incubated under the same conditions as the AK assays specified in the previous section. Trichloroacetic acid was not used to stop the enzymatic activity at the end of the assays with OMPs because reductions of the concentrations of ERY, ROX and SMX were observed when it was added. The enzymatic reaction was assumed to stop once the solid phase extraction (SPE) was performed.

Analytical methods. *Conventional parameters.* The operation of the methanogenic digesters was monitored in terms of the temperature, pH, total suspended solids (TSS), volatile suspended solids (VSS), alkalinity, ammonium, and total and soluble COD.⁴⁴ Biogas production was recorded using Ritter milligas counters (Dr. Ing. Ritter Apparatebau GmbH, Bochum, Germany), and its composition was determined through gas chromatography (HP 5890 Series II). Volatile fatty acids (VFA) were measured individually in a gas chromatograph (HP 5890A) equipped with a flame ionization detector (HP 7637A).

Organic micropollutants analysis. Samples from the MRs were centrifuged at 1880 g for 15 min. The supernatant was pre-filtered (AP4004705, Millipore) and filtered at 0.45 μ m (HAWP04700, Millipore) before performing the SPE with 100 mL samples and 60 mg OASIS HLB cartridges (Waters, Milford, MA, USA).^{4,16} Samples from the enzymatic assays (50 mL) did not require any pre-treatment prior to SPE. To quantify the OMPs sorbed onto the methanogenic sludge, ultrasonic solvent extraction (USE) was conducted before SPE, as described by Gonzalez-Gil et al.⁴ The limits of quantification (LOQ) and recoveries applied to the MRs and the AK assays are shown in Table S3.

Statistical analysis. All of the enzymatic assays with OMPs were conducted in duplicate, and each micropollutant concentration was measured twice (n=4). The results are expressed as mean \pm standard deviation. The significant differences between the enzymatic results were statistically tested by analysis of variance (ANOVA) followed by the Dunnett T3 test for multiple comparisons. The normal data distribution was analyzed with the Shapiro-Wilk test, and the variance homogeneity was analyzed with the Levene test. When the variances were not homogeneous, Brown-Forsythe analysis was used to assess the significant differences. All of the statistical tests were performed at a 5% significance level using the IBM SPSS statistics® software 20.0.

RESULTS AND DISCUSSION

Biotransformation of OMPs during methanogenesis. Figure 2 aims to highlight, in a semi-quantitative representation, the relevance of the methanogenic step on the overall removal efficiencies reported for OMPs during AD of sewage sludge. The methanogenic biomass showed the capacity to biotransform all of the tested OMPs, although the efficiencies varied depending

245 on the compound. For example, SMX, NPX, TMP, OP, NP, FLX, EE2, TCS and the musk
246 fragrances were significantly removed during methanogenesis, while the other compounds had
247 biotransformations of less than 50%.

248 It was not easy to get a single value for the removal of OMPs during AD process due to the
249 divergences on the reported values. Trying to narrow these differences, the average AD removals
250 depicted on Figure 2 correspond to studies that used mainly continuous mesophilic digesters,
251 treating sewage sludge at a HRT between 10 and 30 d (Table S6). Even though, IBP, DCF, BPA,
252 NP, FLX, the hormones and the musk fragrances still presented high deviations, so it was not
253 possible to accurately determine the influence of the methanogenesis on their disappearance.
254 Most of the OMPs appear near the diagonal (between the two dashed lines) of Figure 2,
255 suggesting that the methanogenic biomass is the main responsible for their biotransformation
256 during AD. Therefore, the action of key enzymes participating in the methanogenic route,
257 particularly AK, were further investigated in the next sections. ROX and TMP are the only
258 compounds that clearly showed a higher removal during the overall AD, which indicates that
259 other anaerobic communities (i.e., hydrolytic and acidogenic) widely participate on their
260 removal.

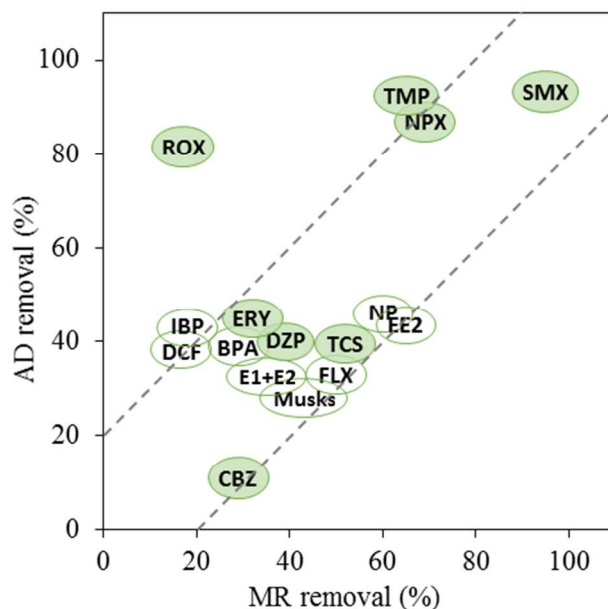


Figure 2. Semi-quantitative representation of the removal efficiencies of OMPs in the methanogenic reactors (MR, x-axis) versus the average removal during AD of sewage sludge^{4,8-12,45-47} (y-axis). Compounds with large divergences on the reported AD removals (standard deviations above 25%) are depicted by open ellipses. No data about the removal of OP in AD was found (60-70% in the MRs). The two dashed lines delimit the region where methanogenesis explains to a high extent the overall AD removal of the OMPs.

Transformation of OMPs in the AK assays. The experiments were performed at two reaction times (1 and 5 days) and, with the exception of the negative control in the absence of AK, consisted of two assays with and without acetate, which were intended to assess the role of the main substrate on the possible AK catalytic transformation of OMPs. No significant differences were found between the negative control and the two AK assays in the 1-day experiment (data not shown), likely because the reaction time was not sufficient to illustrate the action of AK on OMPs. Hence, this section focuses on the 5-day assays.

Performance of assays. The ATP and acetate concentrations during the 5-day assay are shown in Figure 3. The AK activity during the first 2 d was $4.8 \pm 0.2 \text{ U L}^{-1}$, which resulted in a moderate conversion of acetate and ATP (approximately 30%). To increase the enzymatic activity, a second pulse of AK (8.6 mg L^{-1}) and ATP (40 mM) was added to the reaction media after 52 h. As a result, more rapid acetate and ATP consumption was achieved (Figure 3), which led to an average AK activity of $13.4 \pm 1.5 \text{ U L}^{-1}$. After approximately 4 d, the depletion of both reagents almost stopped, which suggests that the phosphorylation of acetate (Reaction 1) reached an equilibrium. This is likely due to the decomposition of hydroxylamine at room temperature in neutralized solutions,^{38,48} which is required to perform Reaction 2 and thus to avoid equilibrium of Reaction 1. This hypothesis is also supported by the fact that the acetate concentration on day 5 was higher than the value that was estimated stoichiometrically from the ATP consumption rates (Figure 3). The average AK activity during the 5-day assay with acetate was much higher (8.0 U L^{-1}) than in the assay without acetate (0.1 U L^{-1}), where a considerably lower conversion of ATP (2% after 5 d) was also achieved because the only potential substrates for AK are OMPs at a much lower concentration than the acetate.

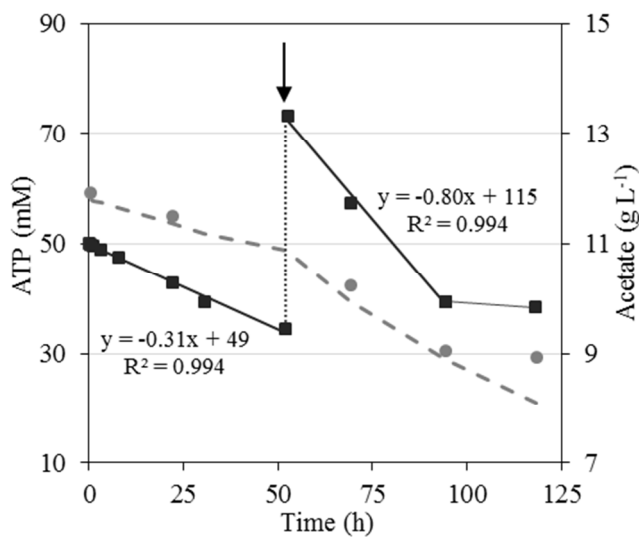


Figure 3. ATP (limiting reagent; squares) and acetate (dots) concentrations in the 5-day AK assay with acetate. The arrow indicates the second addition of ATP and AK. The dashed line represents the stoichiometric acetate concentration considering the consumption rates of ATP.

Chemical OMP transformation. The initial concentration of OMPs in distilled water is compared to the concentration of OMPs after 5 d in the negative control (reaction media without AK) in Figure 4. DZP, AHTN, ADBI, ERY, E1, EE2 and CBZ nearly completely disappear, likely due to the reaction between the ketone group of these compounds (except EE2) with the hydroxylamine required to perform the enzymatic assays. This hypothesis was confirmed experimentally because the OMPs concentration did not decrease in a reaction media without this amine. However, hydroxylamine was included in the reaction media to shift the action of AK towards acetate phosphorylation (Reaction 1). Obviously, this decision masks any possible effect of AK on the biotransformation of these seven OMPs.

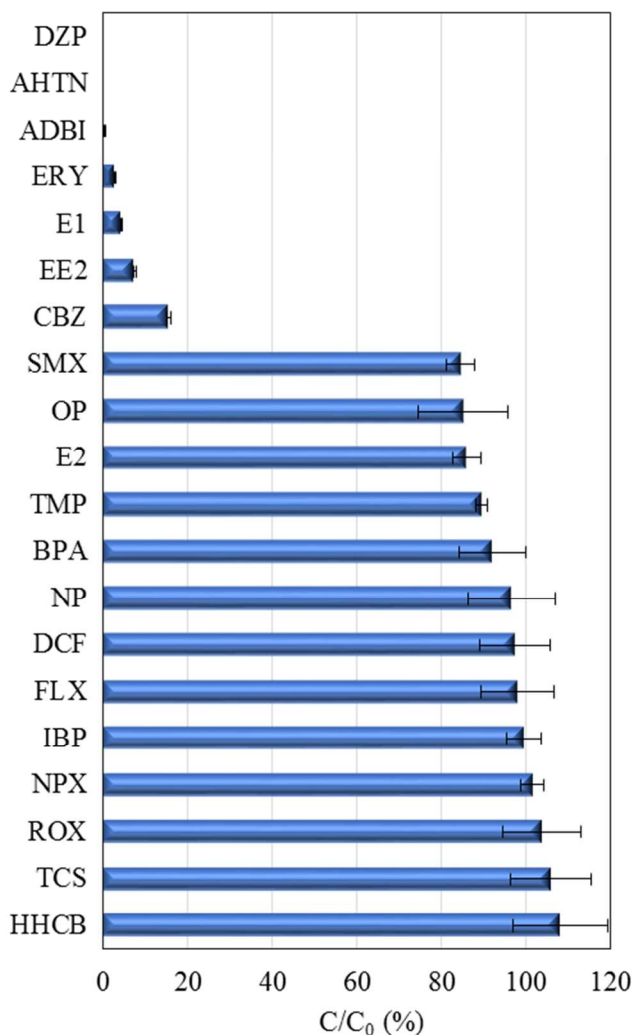


Figure 4. Chemical transformation of OMPs expressed as the ratio between the concentration in the negative control (reaction media without enzyme) after 5 d (C) to the initial concentration in distilled water (C₀).

Enzymatic transformation. The concentrations of the 13 OMPs that were not chemically transformed in the negative control after the 5-day assays with AK are shown in Figure 5. Based on their chemical structures (Table S1), these compounds are classified into three groups. The first group includes carboxylic compounds (IBP, NPX, DCF), the second group are OMPs with hydroxyl groups (NP, OP, BPA, TCS, ROX, E2), and the third group comprises compounds with

other functional groups (HHCB, SMX, TMP, FLX). All data were normally distributed and, except for NPX, OP and HHCB, their variances were homogeneous. FLX, TMP, SMX, E2 and ROX were not affected by AK, since no significant differences were found between the negative control and both enzymatic assays (with and without acetate). In contrast, this difference was statistically significant ($p < 0.05$) for IBP, NPX, DCF, NP, OP, BPA, TCS and HHCB proving that these 8 compounds are transformed by the action of the enzyme. No statistical differences were found between the concentrations of these 8 OMPs in the AK assays performed in the presence and absence of acetate, which suggests that no competitive inhibition occurred between the primary substrate (acetate) and the cometabolic substrates (OMPs).^{19,20} Moreover, these results suggest that once AK is available it can directly transform OMPs in the absence of acetate. However, the presence of the primary substrate is needed to trigger the synthesis of AK during the AD process; therefore, according to the definition of cometabolism,^{17,20,21} the transformation of OMPs (non-growth substrates) is fortuitous and inherently linked to acetate.

To understand how AK can transform these compounds, it is necessary to look into the AK specificity. The action of AK from *M. thermophila* is quite restrictive because the size of the hydrophobic pocket and its affinity to the methyl group of acetate are determinant for substrate specificity.⁴² However, this enzyme still shows slight activity over larger substrates with carboxyl groups (propionate, butyrate), alcohols (ethanol) and even over compounds without hydrophobic groups (formate, glycerol, glycine, glycolic acid).^{29,30} For that reason, IBP, NPX and DCF, which have a carboxyl group (Group 1, Table S1) and are relatively small (1-2 benzene rings), were significantly ($p < 0.05$) biotransformed by AK (10-15%). In addition, compounds with hydroxyl groups in their chemical structure (Group 2, Table S1) could also be appropriate substrates for AK. Nevertheless, the molecular size appears to be determinant

335 because only the smaller compounds (NP, OP, BPA and TCS) were biotransformed (15-32%).
336 The steric hindrance of ROX and E2 could hinder the formation of the enzyme-substrate
337 complex and their further reaction. Similarly, ERY, EE2 and E1, which reacted chemically with
338 the hydroxylamine, would not be affected by AK due to their large molecular size. As expected,
339 the concentrations of the rest of the OMPs (Group 3, Table S1) did not decrease during the
340 enzymatic assays, excluding HHCB (45-50%), for which a clear explanation was not found. It is
341 hypothesized that AK could attack the ether group of this compound, but no references were
342 found to support this enzymatic transformation.

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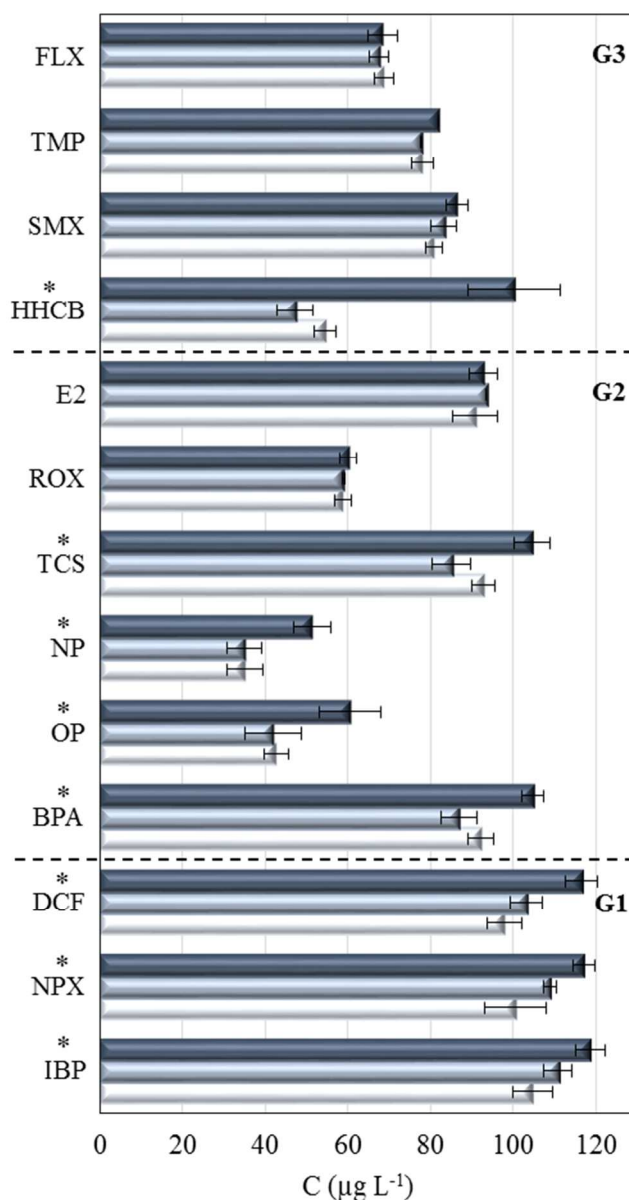
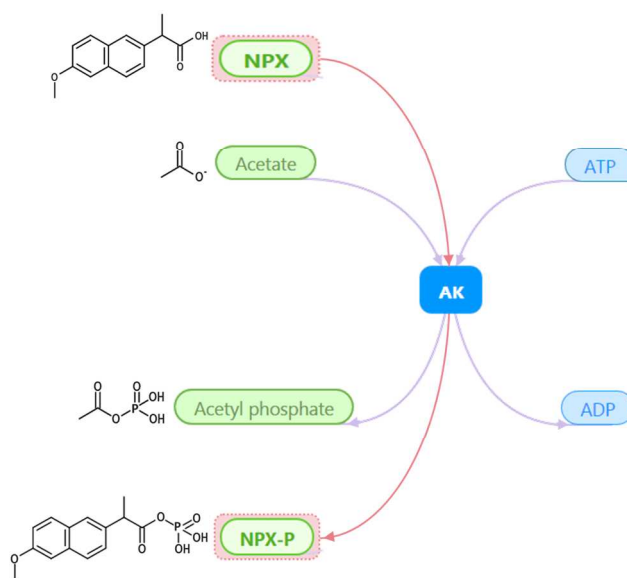


Figure 5. Transformation of OMPs by AK after 5 days of reaction time. The dark bars represent the concentrations of OMPs in the negative control (reaction media without enzyme), the light bars refer to the assay with acetate, and the white bars refer to the assay without acetate. The asterisks indicate the statistical differences ($p < 0.05$) of both AK assays with respect to the control. The compounds are sorted according to their chemical structures in three groups: (G1) OMPs with a carboxyl group, (G2) OMPs with a hydroxyl group and (G3) OMPs with other functional groups.

352 The results of these experiments demonstrate the importance of combining the specificity of
 353 the enzymes and the chemical structure of OMPs to understand the biotransformation
 354 mechanisms. Accordingly, we hypothesized that some of the evaluated OMPs (Figure 5) are
 355 enzymatically transformed by the cometabolic action of AK when ATP is available in the media
 356 and disregarding the presence of the primary substrate (acetate). The proposed pathway predicts
 357 the formation of phosphorylated TPs. For example, the carboxyl group of NPX could act as the
 358 acceptor of a phosphoryl group, as occurs with acetate (Figure 6). Because the detection and
 359 identification of TPs is very challenging, a deep understanding of the cometabolic enzymatic
 360 biotransformation of OMPs allows TPs to be predicted, as was revealed in this study.



361
 362 **Figure 6.** Proposed cometabolic pathway for the biotransformation of some OMPs, such as
 363 NPX, by the enzyme AK during methanogenesis.

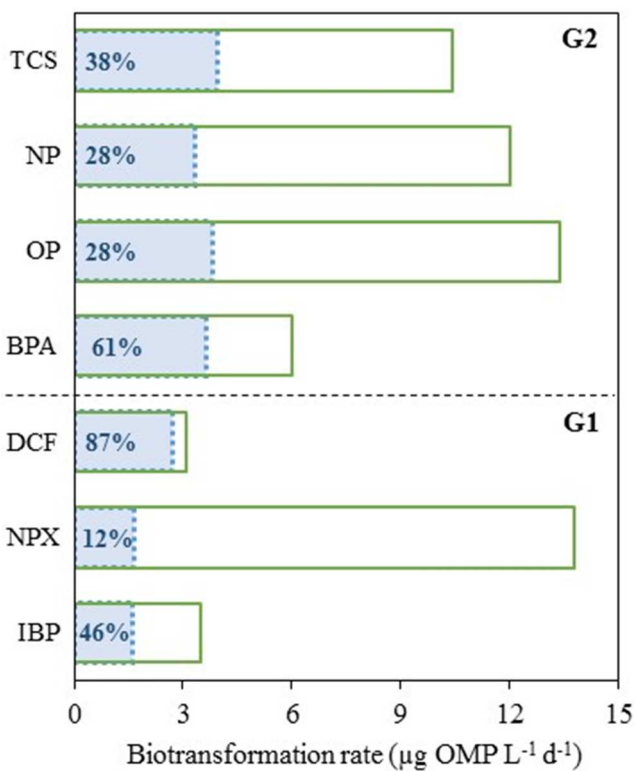
364 **Relevance of AK to the biotransformation of OMPs during methanogenesis.** The
 365 previous section suggests that when sufficient ATP is present in the media, AK could
 366 cometabolically phosphorylate compounds with carboxyl or hydroxyl groups and relatively low
 367 steric hindrance, such as IBP, NPX, DCF, NP, OP, BPA and TCS. Therefore, the next step is to

determine the relevance of this mechanism to the overall biotransformation that was observed for the aforementioned OMPs in the MRs. Figure 7 compares the biotransformation rates ($\mu\text{g L}^{-1} \text{d}^{-1}$) that were obtained in the AK assays with acetate and in the MRs at the same reaction time (5 d). It is important to note that the AK activities in the MRs and the enzymatic assay are quite similar (7.0 and 8.0 U L^{-1} , respectively). Assuming that 2/3 of the total CH_4 is produced from acetate by aceticlastic archaea,²⁶ the theoretical AK activity in the MRs (OLR of 1 g COD $\text{L}^{-1} \text{d}^{-1}$ and 70% methanization would be 10 U L^{-1} , which is similar to the measured value.

Depending on the compound, the biotransformation rates in the MRs varied from 3.0 to 14 $\mu\text{g OMP L}^{-1} \text{d}^{-1}$, while this range is narrower in the AK assays (1.5–4.0 $\mu\text{g OMP L}^{-1} \text{d}^{-1}$). IBP and DCF are hardly biotransformed during methanogenesis (3.0–3.5 $\mu\text{g OMP L}^{-1} \text{d}^{-1}$), although an important removal mechanism (>45%) is related to AK action. The AK cometabolic mechanism is also relevant (61%) in the methanogenic biotransformation of BPA (6 $\mu\text{g OMP L}^{-1} \text{d}^{-1}$). In contrast, the transformation of NPX by AK (12%) does not explain its high depletion during methanogenesis (14 $\mu\text{g OMP L}^{-1} \text{d}^{-1}$). Likewise, AK accounts for only 28–38% of the overall biotransformation observed for OP, NP and TCS in the MRs (10–13 $\mu\text{g OMP L}^{-1} \text{d}^{-1}$). On the other hand, AK would explain the whole biotransformation rate of HHCB in the MR (9 $\mu\text{g OMP L}^{-1} \text{d}^{-1}$); what is a quite surprising fact, especially because it cannot be ascertain that this transformation occurs via the proposed cometabolic phosphorylation pathway.

Based on these results, no direct relationship was found between the degree of biotransformation during methanogenesis and the contribution of the AK cometabolic mechanism. Moreover, the biotransformation rate was always higher in the MRs, which suggests that other active enzymes in addition to AK could further transform them. Some candidate enzymes could be those that continue the aceticlastic methanogenesis (Figure S1), those involved

in the hydrogenotrophic methanogenesis⁴⁹ or other kinases, such as propionate and butyrate kinase. These two kinases have a larger hydrophobic pocket than AK to directly phosphorylate propionate and butyrate, respectively;^{50,51} therefore, they could be involved in the enzymatic transformation of the same OMPs as AK, but they could also promote the transformation of larger OMPs with hydroxyl groups, such as ERY, ROX, E1, E2 and EE2, which were not affected by AK. Otherwise, the high methanogenic biotransformations of TMP and SMX (Figure 2) should be caused by other types of enzymes because kinases will not modify their chemical structure. In summary, by combining enzyme action and the OMP's chemical structure, new insights are provided to understand the transformation mechanisms of OMPs during AD, which is useful for developing new strategies to maximize the elimination of OMPs from sewage sludge and to predict the TPs that form and their potential risks.



403 **Figure 7.** Biotransformation rates of the OMPs prone to be cometabolically phosphorylated by
404 AK in the MRs (empty green bars) and in the AK assay with acetate (blue-pointed bars) after 5
405 days. The percentages indicate the contribution of AK to the methanogenic biotransformation.
406 G1 refers to OMPs with carboxylic compounds, and G2 refers to OMPs with hydroxyl groups.
407 HHCB was excluded from this figure due to the lack of information regarding the
408 phosphorylation action of AK on ether groups.

409 ASSOCIATED CONTENT

410 Supporting Information

411 The Supporting Information is available free of charge on the ACS Publications website.

412 Enzymatic pathways of the acetoclastic methanogenesis (Figure S1); chemical structures (Table
413 S1) and physicochemical properties (Table S2) of the OMPs; limits of quantification and
414 recoveries of the analytical methods (Table S3); details of the MRs performance (section S4,
415 Table S4); details of the acetate kinase activity determination (section S5, Figure S2); results
416 from the preliminary assays with AK (Table S5) and summary of the removal of OMPs during
417 AD (Table S6).

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422 Notes

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